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REPORTS

DNA Measurements by Single Nuclei Flow Cytometry in Human Actinic Skin Lesions

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A basal cell carcinoma, a Bowenoid carcinoma and clinically normal nonexposed skin from a 76-yr-old male with numerous epidermal tumors on sun-damaged skin were investigated by DNA flow cytometry and the results were compared with data obtained from normal skin of a control group.

From each type of skin lesion 3 different biopsies were examined. Each of the DNA frequency distribution histograms showed bimodal configuration revealing no more than one DNA stem line, which was diploid in the unaffected skin and the Bowen carcinoma and hyperdiploid in the basal cell carcinoma.

The 3 different types of skin could be clearly separated by means of at least 1 of 3 parameters: S-fraction, G_2 + M fraction or S/G_2 + M index. The S/G_2 + M index came out as the most sensitive discriminator for the tumors. The S-fraction level was higher in the clinically normal skin of the investigated male than in the control group and further high in the tumors.

In recent years single cell DNA flow cytometry has yielded valuable information supplementary or alternative to those obtained by conventional cell kinetic methods for several cell systems [1–3]. We have recently described a modified technique for single nuclei DNA flow cytometry on human skin [4]. For ethical reasons *in vivo* autoradiographic investigations are difficult to perform in man. Hence, the value of measurements of single cell DNA-content by flow cytometry has to be judged either by comprehensive methodological studies on variability, reproducibility and clinical relations or by comparative *in vivo* studies, i.e., on cells with fundamentally changed external conditions. Premalignant and malignant epidermal tumors in man infrequently grow to a size which permits histologic evaluation as well as multiple biopsies for studies on cell kinetic. We have had the opportunity to study 2 large epidermal tumors in one individual. The main aim of this study was to estimate the variability and mitotic counting in several large biopsies in different skin tumors in this individual to provide a rational basis for future relevant use of single biopsy results.

MATERIALS AND METHODS

A male, aged 76, with an uneventful chronic lymphatic leukemia controlled on prednisone 5 mg a day with multiple superficial skin tumors, mainly on light exposed areas, was studied. A previous arsenic intake by ingestation of ironcontaining drugs and tonics was likely, but not provable. Although his skin usually reacted strongly with erythema and scaling to sun exposure, he had sunbathed extensively during his whole life and when possible by exposing his entire integument.

His whole skin surface bore a stamp of sun damage, and unnumerable small superficial keratoses were present, as well as larger tumors, some ulcerating, on the upper part of the body, the legs, the face, neck, and crown.

The main part of the more than 20 tumors were actinic keratoses histologically. Eight basal cell carcinomas, 2 squamous cell carcinomas, 1 Bowen carcinoma, 1 keratoacanthoma, and 1 clear cell acanthoma were removed. Two of the tumors were investigated by DNA flow cytometry, i.e., a centrally slightly ulcerated superficial tumor measuring 2.5 × 5 cm on the upper back, histologically a basal cell carcinoma, and a superficial well demarcated, desquamating lesion measuring 3 × 3 cm in the right lumbal region, histologically a Bowenoid carcinoma.

In each of these tumors 3 biopsy areas were chosen randomly in the peripheral region, all a minimum of 2 cm apart. From each biopsy area 3 × 4-mm punch biopsies were taken. One of the 3 adjacent biopsies was used for histology after treatment in Bouin-alcohol. The remaining 2 biopsies were pooled and used for determination of the 2c, 2–4c and 4c DNA cell fractions according to a standard procedure [4].

Furthermore 3 surgical biopsies measuring approximately 1 × 1.5 cm were taken a minimum of 2 cm apart from the lower abdomen, which was less sun-exposed and appeared clinically normal.

The biopsies meant for flow cytometry were immediately placed in 1% acetic acid for maceration by 4°C and 2 days later stored in liquid nitrogen without any medium. When finally used the tissue was thawed and the cells in the samples separated by treatment with dithiotreitol and ultrasonication as previously described [4]. The resulting single nuclei suspension was incubated with RNA-ase before staining by ethidium bromide. Finally the DNA content of each nucleus was measured in a flow cytometer combined with a multichannel analyzer (cytofluorograph, BioPhysics System Inc. Type 4802A and 6300A) and the DNA distribution for each sample was recorded as a histogram (Fig. 1). About 100,000 cells were measured in each sample. The DNA histograms were statistically analyzed by a computerized maximum likelihood estimation procedure quite corresponding the least squares calculations previously described [4]. The final results expressed the fraction of nuclei with a 2c DNA content ('G_1' cells, in normal epidermis both G_1, G_0 and differentiated cells), with a 2–4c DNA content (S-cells, in normal epidermis DNA synthesizing cells), and with a 4c DNA content (G_2 + M cells, the sum of cells in G_2 phase and mitoses).

The G_1 peak for the nuclei from a sample of normal skin was regarded as representing the diploid cell population. The ploidy of the tumors was estimated from the position of the channel number for the G_1 peaks, the stability of the fluorescence signals controlled by run of standard fluorescent particles before and after the skin sample measurements.

The results were compared with values from seven anatomically adjacent skin samples in a control person without skin diseases and additionally with values from abdominal skin from 10 control persons in a comparable age-group.

RESULTS

The results are graphically summarized in Fig. 2. For the unaffected skin of the patient the fraction of S-phase cells was higher than the S-fraction in a group of lo control persons ($p < 0.01$, Student's t-test). The G_2 + M-fraction, in contrast, did not differ significantly from the inter-individually rather variable G_2 + M-fractions in the control groups.

Both the basal cell carcinoma and the Bowenoid carcinoma exhibited higher 2–4c DNA fractions than did the clinically normal skin of the patient, yet the increase was only statistically

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significant for the basal cell carcinoma ($p < 0.05$, approximate t-test). The Bowenoid carcinoma on the other hand differed significantly from the clinically normal skin by a high 4c DNA fraction ($p < 0.01$, t-test). For both tumors as well as the 2–4 c as the 4c DNA fractions were significantly higher than in the control group.

In the 3 samples from each of the tumors the variability in results exceeded the measurement error in normal skin, but only for the S-fraction significantly on the 5% probability level (F-test). The corresponding $S/G_2 + M$ indices were rather stable with differing levels for Bowen carcinoma and the basal cell carcinoma.

The cell population in the basal cell carcinoma was estimated as hyperdiploid, as the G_2 peaks for the 3 samples varied from channel number 51–55 and the G_2 peaks for the 3 samples from the unaffected skin varied from channel 37–41 ($p = 0.001$). The Bowenoid carcinoma had no aneuploid cell population by analogous analysis ($p > 0.2$). For each of the skin conditions bimodal DNA distributions were seen, and thus no evidence for the presence of more than one DNA stem line in each sample was found.

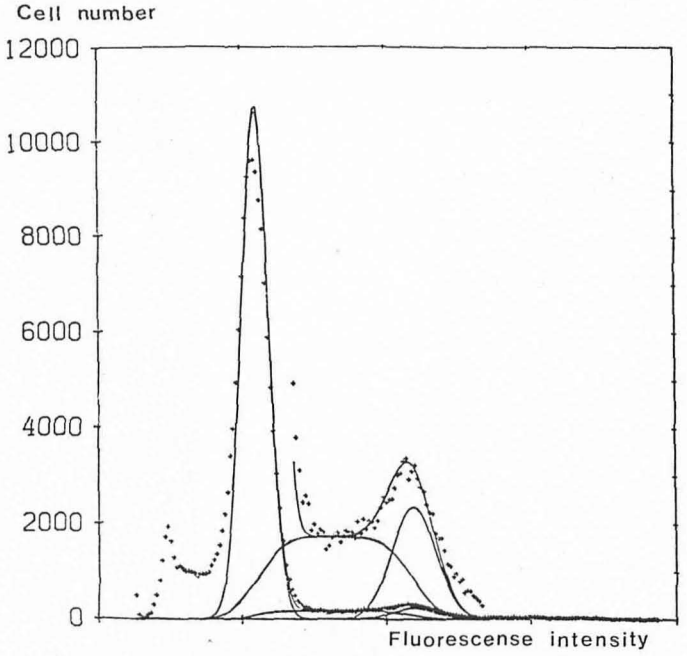

FIG 1. shows the DNA-frequency distribution of the basal cell carcinoma investigated as measured by single nuclei flow cytometry. The fluorescence intensity of the single nuclei (*abscissa*) parallels the DNA-content. The *upper solid line* at the right represents a 10-fold amplification of the number of cells (nuclei) given by the *lower solid* line. The area below the first maximum represents the number of cells with a 2c DNA content (G_1(G_0-fraction and differentiated cells), the area below the second maximum the number of cells with a 4c DNA-content (G_2 + M-fraction) while the intermediate area represents the number of cells with 2–4c DNA content (S-fraction). For the 2 latter fractions as well the areas by size corresponding the G_1-area (*lower solid lines*) as the 10 times amplified areas (*upper solid lines*) are given. The range of the coefficients of variation for the G_1-peak for the samples was 7.6–9.9%.

DISCUSSION AND CONCLUSIONS

Reports on photometric measurements of the nuclear DNA content in human tumors are previously given by Kint [5], Ehlers [6], Ehlers and Stephen [7,8], Ehlers and Herbstreit [9], the measurements performed on Feulgen-stained tissue sections and concerning nuclei in all cell cycle phase. Manocha [10], Manocha, Steele, and Stich [11] used a similar technique, but considered only metaphase nuclei for optimal evaluation of the tumor stem cell lines and ploidy. However, the technique of histophotometric DNA measurements is extremely laborious, so most results are based on measurements of only 50–100 nuclei/sample. Further, it may be difficult to avoid errors from sectioned nuclei. When automatized DNA-measurements on suspensions of single nuclei had become possible yielding the advantage of rapid analysis of a very large number of nuclei, Schumann, Ehring, Göhde, and Dittrich in 1971 preliminarily presented the use of the method on a limited number of human basal and squamous cell carcinomas [12].

The reports hitherto primarily have concerned the very tumors, each generally described by one joint DNA frequency distribution. In the present study we have 3 different DNA determinations for each lesion. Additionally we have studied the unaffected skin in this individual developing multiple epidermal tumors, presumably as a result of exposure to two wellknown epidermal carcinogens, i.e., arsenic intake and heavy sunbathing. Although our results arise from only one individual

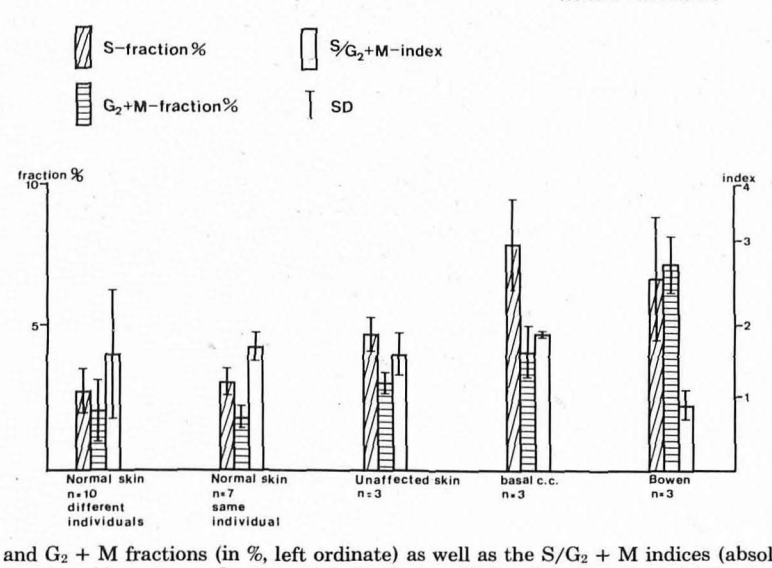

FIG 2. compares the S- and G_2 + M fractions (in %, left ordinate) as well as the S/G_2 + M indices (absolute values, right ordinate) and the variability in these parameters in epidermal samples from 10 individuals suffering from leg ulcers (abdominal skin), and in 7 samples from one healthy person (femoral skin) with the nontumorous lower abdominal epidermis, the basal cell carcinoma (basal c.c.) and the Bowenoid carcinoma from the investigated male.

certain trends in the DNA flow-cytometric parameters in sun-damaged human skin do emerge. The S-fraction in the unaffected skin of this multiple epidermal carcinoma patient was significantly higher than in analogous skin samples from a control group. Additionally high S-fractions were found in the tumors. This might suggest, that an unusual high S-fraction in clinically normal, unstimulated skin should draw attention to a potential propensity for malignant transformation. Supplementary studies should elucidate this possibility (Frentz and Møller, forthcoming).

In the previously mentioned report Kint stated elevated mean DNA-contents in skin adjacent to squamous cell carcinoma and skin covering but not in skin adjacent to basal cell carcinoma [5]. Epidermis adjacent to actinic keratoses is reported to exhibit increased values by ^{3}HTdR labelling [13].

The results in the present study suggest that single punch biopsies from the peripheral region of a skin tumor might yield results rather representative for the proliferative activity of the entire periphery. The parameters measured within each tumor were sufficiently stable to allow distinction between the neoplastic lesions by means of the G_2 + M fraction and the S/G_2 + M index. No evidence for more than one stem line within the tumors was seen, this in accordance with the homogeneity in the S- and G_2 + M values. A highly aggressive skin tumor, the malignant melanoma, in contrast recently has been reported to exhibit a so pronounced heterogeneity that single biopsies not can be regarded as representative for the tumor [14].

The aneuploidy of the invasive, yet clinically not aggressive basal cell carcinomas seems to be a rather sufficient, but not an indispensible marker of its malignancy, as only about one-fourth of basal cell carcinomas should be characterized by this feature as recently reported by Schumann, Göhde, Barlogie, and Kreidler [15].

As for the interpretation of the parameters measured by flow cytometry it must be stressed that a high S-fraction per se not can be regarded as a feature specific for virtual or potential malignancy.

The S-fraction is just a figure giving the proportion of cells in S-phase at the biopsy sampling moment. A rise in the S-fraction may be due to prolongation of the mean S-phase transit time, increased S-influx or decreased S-efflux as well as to these features in combination. This implies that a high S-fraction just may reflect a simply increased growth fraction. A high S-fraction or G_2 + M fraction and a contemporary change in the S/G_2 + M index are, however, likely to signify intrinsic alteration in the proliferative compartment, i.e., shifting in the proportion of cells in the various cell cycle stages. The S/G_2 + M index in itself has no well-established or univocal cell kinetic meaning, yet the emperical use of the parameter might prove helpful in further studies as suggested from the present results.

These considerations are important for the interpretation of differences in flow cytometric parameters, not least between tumors and their precursor tissue.

Niels Keiding, Statistical Research Unit, Danish Medical and Social Research Councils, surveyed the statistical calculations, Kirsten Flod and Solveig Vesterlund performed the technical work.

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